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RESEARCH ARTICLE

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Hepatic E4BP4 induction promotes lipid accumulation by suppressing AMPK signaling in response to chemical or diet-induced ER stress

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Abstract

Prolonged ER stress has been known to be one of the major drivers of impaired lipid homeostasis during the pathogenesis of non-alcoholic liver disease (NAFLD). However, the downstream mediators of ER stress pathway in promoting lipid accumulation remain poorly understood. Here, we present data showing the b-ZIP transcription factor E4BP4 in both the hepatocytes and the mouse liver is potently induced by the chemical ER stress inducer tunicamycin or by high-fat, low-methionine, and choline-deficient (HFLMCD) diet. We showed that such an induction is partially dependent on CHOP, a known mediator of ER stress and requires the E-box element of the *E4bp4* promoter. Tunicamycin promotes the lipid droplet formation and alters lipid metabolic gene expression in primary mouse hepatocytes from *E4bp4*$^{flox/flox}$ but not *E4bp4 liver-specific KO* (*E4bp4-LKO*) mice. Compared with *E4bp4*$^{flox/flox}$ mice, *E4bp4-LKO* female mice exhibit reduced liver lipid accumulation and partially improved liver function after 10-week HFLMCD diet feeding. Mechanistically, we observed elevated AMPK activity and the AMPKβ1 abundance in the liver of *E4bp4-LKO* mice. We have evidence supporting that E4BP4 may suppress the AMPK activity via promoting the AMPKβ1 ubiquitination *and* degradation. Furthermore, acute depletion of the *Ampkβ1* subunit restores lipid droplet formation in *E4bp4-LKO* primary mouse hepatocytes. Our study highlighted hepatic E4BP4 as a key factor linking ER stress and lipid accumulation in the liver. Targeting E4BP4 in the liver may be a novel therapeutic avenue for treating NAFLD.

Abbreviations: ACC1, acetyl-CoA carboxylase 1; ALT, alanine aminotransferase; AMPK signaling, AMP-activated protein kinase signaling; ATF4, activating transcription factor 4; ATGL, adipose triglyceride lipase; C/EBP β, CCAAT/enhancer-binding protein β; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homologous protein; CIDEC, cell death-inducing DFFA-like effector C; CPT1A, carnitine palmitoyltransferase 1A; CREBH, cyclic adenosine monophosphate (cAMP)-responsive element-binding protein H; E4BP4, E4 promoter-binding protein 4; ER stress, endoplasmic reticulum stress; FASN, fatty acid synthase; FXR, farnesoid X-activated receptor; GLP, glucagon-like peptide; HFLMCD diet, high-fat, low-methionine, and choline-deficient diet; MKRN1, makorin ring finger protein 1; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PLIN4, perilipin 4; PMHs, primary mouse hepatocytes; SCD1, stearoyl-Coenzyme A desaturase 1; TG, triglycerides; UPR, unfolded protein response; XBP1, X-box binding protein 1.

[Correction added on September 7, 2020, after first online publication: Figures 6 has been replaced with revised figure.]

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KEYWORDS

de novo lipogenesis, ER stress, high-fat, low-methionine, and choline-deficient diet, lipid accumulation, lipid droplet

1 | INTRODUCTION

The ER system is an intracellular organelle responsible for the folding of membrane-bound and secreted proteins, lipid and sterol synthesis, and calcium storage in eukaryotes.[1,2] When unfolded or misfolded proteins accumulate inside that ER lumen, ER stress triggers a group of signal transduction pathways namely "unfolded protein response (UPR)" to reprogram the transcriptional and translational processes for recovery or adaptation. A variety of pharmacological agents and dietary manipulations have been shown to cause ER stress and interrupt the normal ER function.[3,4] One of the metabolic consequences of ER stress in hepatocytes is lipid accumulation in the liver. Acute injection of the ER stress inducer tunicamycin in mice was shown to not only induce the UPR genes but also cause marked lipid droplet accumulation along with increased de novo lipogenesis and reduced fatty acid oxidation in the liver.[5] Methionine and choline deficient (MCD) diet, which is known to induce liver steatosis and injury, induces potent ER stress in the liver.[6] These findings highlighted that ER stress might be a critical regulator of hepatic lipid accumulation, the early hallmark of non-alcoholic fatty liver disease (NAFLD).

The precise mechanisms of NAFLD remain poorly understood. Currently, the "multiple-hit hypothesis" has been recognized to address the development and progression of NAFLD.[7,8] The initial hit leads to simple liver steatosis, while oxidative stress, insulin resistance, impairment in lipid metabolism, and Kupffer cell activation result in hepatic inflammation and apoptosis, prompting the progression from simple steatosis to non-alcoholic steatohepatitis (NASH).[9] Hepatocytes are rich in ER due to a high capacity for handling protein and lipid biosynthesis. It has been hypothesized that impaired ER homeostasis may be directly involved in the onset and progression of NAFLD.[2,10] For example, the markers of ER stress were elevated in the liver of NAFLD.[6,11,12] Chemical chaperons have been shown to not only reduce ER stress but also improve liver function.[13,14] Deletion of the ER stress responsive chaperon $p58^{IPK}$ protects mice from diet-induced steatohepatitis.[15] Several drugs including FXR agonists and GLP analogs have shown great promise in treating NAFLD/NASH through potent inhibition of the ER stress pathways in the liver.[16,17]

There has been an intense interest in the role of transcription factors in the pathogenesis of NASH, particularly the ER-stress associated transcription factors, such as CHOP, ATF4, XBP1, CREBH, and C/EBP-β.[8] The Friedman group showed that deletion of *C/ebp-β* attenuates inflammation and lipid accumulation in diet-induced NASH.[18] In contrast, *Chop* deletion results in more severe NASH following diet high in fat, fructose, and cholesterol.[18,19] Hepatic *Xbp1* deficiency sensitizes mice to diet-induced NASH.[20] *Crebh* deficiency also accelerated the development of NAFLD in mice fed atherogenic diet.[21] These paradoxical results demonstrated the diverse functions of ER stress-associated transcription factors in the pathogenesis of NAFLD. Thus, a better understanding of the specific roles for individual components of the ER stress pathway may lead to effective treatment for NAFLD.

As a b-ZIP transcription factor, E4BP4 is ubiquitously expressed in various tissues, particularly abundant in the liver, spleen, and adipose tissue.[22] Although E4BP4 is well-known for its critical role in NK cell development and lymphocytes[23,24] and has been recently implicated in hepatic gluconeogenesis and the regulation of body composition,[25,26] the involvement of E4BP4 in lipid metabolism remains largely unexplored. We previously reported that potent induction of E4BP4 by insulin contributes to the postprandial de novo lipogenesis in an SREBP-1c-dependent manner.[27] *E4bp4*-deficient hepatocytes exhibit a significant reduction in de novo lipogenesis, triglyceride content as well as the expression of lipogenic enzymes including *Fasn* and *Scd1*. The work from the Zhang group shows that E4BP4 could impact hepatic lipid metabolism via its interaction with CREBH, a liver-enriched ER-tethered transcription factor known to regulate hepatic lipid homeostasis.[21] All these findings support a physiological role of E4BP4 in regulating hepatic lipid metabolism. Because dysregulated lipid metabolism is a major contributor to the pathogenesis of NAFLD, we set out to study the role of E4BP4 in this process. In the present study, we report that E4BP4 is highly induced by either chemical or diet inducers of ER stress in hepatocytes and the liver. *E4bp4*-deficient hepatocytes are resistant to tunicamycin-induced lipid accumulation. Moreover, the liver from *E4bp4-LKO* mice on NASH-inducing HFLMCD diet show decreased lipid accumulation, improved lipid metabolism, and reduced liver injury along with the enhanced AMPK signaling. Our data demonstrated that hepatic E4BP4 may drive the pathogenesis of diet-induced NAFLD likely through its inhibitory effects on the AMPK pathway.

2 | MATERIALS AND METHODS

2.1 | Animal and treatment

Animal experiments were conducted in accordance with the guidelines of the institutional Animal Care and Use

Committee of University of Michigan Medical School. Male *C57BL/6J* mice and *Albumin-Cre* mice were purchased from *the Jackson Laboratory*. *E4bp4*$^{flox/flox}$ mice were generated via the conventional homologous recombination targeting exon 2 of the trimouse *E4bp4* at *Cyagen* (Santa Clara, CA). Liver-specific *E4bp4* knockout (*E4bp4-LKO*) mice were generated by crossing *E4bp4*$^{flox/flox}$ mice with *Albumin-Cre* mice. All mice were housed on a 12 hours:12 hours light/dark cycle at 25°C with free access to water and regular chow (26.8% kcal from protein, 16.6% from fat, and 56.4% from starch). At the age of 2 months, *E4bp4* $^{flox/flox}$ and *E4bp4-LKO* female mice were fed high-fat, low-methionine, and choline-deficient (HFLMCD: 45% calories from fat, 0.1% methionine, and choline-deficient) diet for 10 weeks. For *Ampk-β1* knockdown experiment, 2-months-old WT mice were fed HFLMCD diet for 2 weeks, and then, injected with adenoviral shRNA against *LacZ, E4bp4,* or *E4bp4* plus *Ampk β1* through tail vein. After injection, mice continued with HFLMCD diet for another 10 days prior to liver harvesting for metabolic assays.

2.2 | Cell cultures

Hepa1c1c-7 cell was purchased from the ATCC and maintained according to the instructions. Isolation of primary mouse hepatocytes (PMHs) was described previously.[27] PMHs and Hepa1 cells were treated with DMSO and 5 μM tunicamycin for 24 hours and harvested for western blotting, RT-qPCR, and BODIPY staining.

2.3 | Liver Histology and Sirius-Red staining

Liver tissues were immediately fixed in 10% formalin at room temperature overnight after paraffin embedding and H&E staining. While the H&E-stained slides were observed directly under microscope, the unstained slides were baked in an oven set at 58ºC for 1 hour. And the paraffin sections were rehydrated in ethanol solutions in various concentrations. Then, the slides were stained with Picro-Sirius Red Collagen Stain Kit (Sigma, Direct Red 80, 365548; FAST Green, 7252) or Masson's Trichrome Stain Kit (Sigma, HT15-1KT) according to the user's manuals and observed under microscope.

2.4 | Transfection and luciferase assay

A total of 2 x 10^5 293AD cells were seeded in 24-well plates. Transfection was performed with Opti-MEM (Gibco, 2021431) and polyethylenimine (PEI). The β-gal expression plasmid and *Chop-HA* over-expression plasmids were co-transfected with the *E4bp4 WT promoter or the E4bp4 ΔE-box* promoter-driven luciferase reporter construct plus the *β-gal* expression plasmid. Twenty-four hours later, the cells were harvested for luciferase and β-gal assays. Luciferase assay was performed with Luciferin (Gold Biotechnology, 103404-75-7) and normalized by the β-gal signal.

2.5 | Protein extraction and immunoprecipitation

To prepare cytosolic and nuclear proteins, liver tissues were homogenized in hypotonic buffer, incubated on ice for 15-20 minutes, and centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was saved as the cytosolic fraction. The pellet was washed once with hypotonic buffer and re-suspended in RIPA buffer prior to sonication for 5 seconds. The nuclear fraction was then collected after centrifugation at 13 000 × rpm for 10 minutes. Western blot analysis was performed using the following primary antibodies: anti-GAPDH (Santa Cruz, sc-25778), anti-Lamin A/C (Santa Cruz, sc-20681), anti-HSP90 (Santa Cruz, sc-13119), anti-CHOP (Santa Cruz, sc-575), anti-GRP78 (Santa Cruz, sc-13968), anti-E4BP4 (Santa Cruz, sc-28203; Cell Signaling, 14312S; DSHB,PCRP-NFIL3-2B5),anti-AMPK-p^{T172}(CellSignaling, 2531S), anti-AMPK α1/α2 (Cell Signaling, 5831S), anti-AMPK-β1 (Santa Cruz, sc-100357), anti-AMPK-γ1 (Santa Cruz,sc-19138),anti-β-tubulin(Sigma,T5201),andanti-FLAG (Sigma, A8592).

2.6 | cDNA Synthesis and qPCR

Total cellular RNA extraction was performed with TRIzol (Invitrogen) and chloroform. cDNA was synthesized with the Verso cDNA kit (Thermo Fisher Scientific) and subjected to qPCR analysis with Radiant Green 2X qPCR Mix (Alkali Scientific) on an ABI 7900 HT thermal cycler (Applied Biosystems). The value of each cDNA was calculated using the ΔΔCT method and normalized to the values of the housekeeping gene control, the 18s ribosomal RNA. The data were plotted as fold change. The primer sequences are listed below (Table 1).

2.7 | BODIPY staining

Primary mouse hepatocytes (PMHs) were isolated from *E4bp4* $^{flox/flox}$ and *E4bp4-LKO* mice using a previously reported protocol. A total of 8 × 10^4 cells were seeded per well of 12-well plates. Next day, cell culture medium was changed to serum-free DMEM (*Gibco*) and the cells were treated with

TABLE 1 qPCR primer sequences

	Forward	Reverse
18S	TTGACGGAAGGGCACCACCAG	GCACCACCACCCACGGAATCG
Acc1	GAAGCCACAGTGAAATCTCG	GATGGTTTGGCCTTTCACAT
Acox1	TGCTGCAGACGGCCAGGTTC	GGCCAGACTGCCACCTGCTG
Acox2	CCTTTGCCCAACGACACTGGCA	ACCGGGAGGTACCAAGAACCTCTG
Acsl3	GCAGCTGCGTCAGGGTCC	TAAGACCCGCGGGCTCCG
Atg12	GGCCTCGGAACAGTTGTTTA	CAGCACCGAAATGTCTCTGA
Atg2a	CACTCTACGCCACTACAT	ATCCAGCACATCCAAGAA
Atg7	CAGAAGAAGTTGAACGAGTA	CAGAGTCACCATTGTAGTAAT
Atgl	TTCACCATCCGCTTGTTGGAG	AGATGGTCACCCAATTTCCTC
Bax	GATCAGCTCGGGCACTTTAG	TTGCTGATGGCAACTTCAAC
Bcl-2l1	CGGATTGCAAGTTGGATGGC	TCAGGAACCAGCGGTTGAAG
Beclin1	GGCCAATAAGATGGGTCTGA	CACTGCCTCCAGTGTCTTCA
Bim	CGGTCCTCCAGTGGGTATTT	TATGGAAGCCATTGCACTGAGA
Cd36	CCAAGCTATTGCGACATGATT	CCGAACCACAGCGTAGATAGACC
Cgi58	CTTGCTTGGACACAACCTG	GAGGTGACTAACCCTTGATGG
Chop	CTGCCTTTCACCTTGGAGAC	CGTTTCCTGGGGATGAGATA
Cidea	ACAGAAATGGACACCGGGTAG	TGACATTGAGACAGCCGAGG
Col1a1	GAGGCCTCCCCAGAACATCAC	CGATCTCGTTGGATCCCTGG
Col1a2	AGTCGATGGCTGCTCCAAAA	AGCACCACCAATGTCCAGAG
Cpt1a	TCTGCATGTTTGACCCAAAA	TTGCTGGAGATGTGGAAGAA
Crbn	TCCTTTGCGGGTAAACAGACA	TCGGTTTTCTGGCTTCTTTACTA
Cyp4a10	GGAGCTCCAATGTCTGAGAAGAGT	TCTCTGGAGTATTCTTCTGAAAAAGGT
Cyp4a14	TCTCTGGCTTTTCTGTACTTTGCTT	CAGAAAGATGAGATGACAGGACACA
Dhcr7	ATTGAGTTCAACCCCCGCAT	AACACGTAGATGGCCTGCAA
E4bp4	ATGGGAAGGCTCTTTCTCCACT	TACCCGAGGTTCCATGTTTC
Elovl7	GCCAAGAGCAATGAGGATGG	GGTCCACGGCATGATCGTAT
Fasn	TTGGCCCAGAACTCCTGTAG	CTCGCTTGTCGTCTGCCT
Fsp27α	GCCACGCGGTATTGCCAGGA	GGGTCTCCCGGCTGGGCTTA
Fsp27β	GTGACCACAGCTTGGGTCGGA	GGGTCTCCCGGCTGGGCTTA
Gadd45a	TGGTGACGAACCCACATTCA	CGGGAGATTAATCACGGGCA
Grp78	GGTGCAGCAGGACATCAAGTT	CCCACCTCCAATATCAACTTGA
Hmgcr	CACAATAACTTCCCAGGGGT	GGCCTCCATTTAGATCCG
Hmgcs	AGAAATCCCTGGCTCGCTTG	AGCTTTAGACCCCTGAAGGC
Hrd1	TTTTCGGCCTGTCAGATGGC	GGCCCAGAGACCTGTGAACG
Hsl	ACGCTACACAAAGGCTGCTT	TCGTTGCGTTTGTAGTGCTC
Idi1	GACGTCACCCTTGTGCTAGA	TAGAACAGAGATTCCGGCTG
Il-1β	TGCAGCTGGAGAGTGTGGATCCC	TGTGCTCTGCTTGTGAGGTGCTG
Il-6	GACAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTG
Lpl	AGAAGGGAAAGGACTCAGCAG	TCAAACACCCAAACAAGGGTA
Lss	ATCCAAGCACTGTTAGAGGCAGGT	TCCAGTGTGCTGAAGGAGAAACCA
Magl	GAACAAGTCGGAGGGTTCTGC	GAGGACGTGATAGGCACCTT
Mcp1	ACTGAAGCCAGCTCTCTCTTCCTC	TTCCTTCTTGGGGTCAGCACAGAC
Mkrn1	GCGAGAAAGGAGATCCGACC	5‘-TTCGTATCTGCAGCGGTCTC
Mmp2	AACGGTCGGGAATACAGCAG	CCACCCATGGTAAACAAGGC

(Continues)

TABLE 1 (Continued)

	Forward	Reverse
Mttp1	CTCCACAGTGCAGTTCTCACA	AGAGACATATCCCCTGCCTGT
Noxa	TGGAGTGCACCGGACATAAC	TCGTCCTTCAAGTCTGCTGG
Pcsk9	CACAATGTAGGTTCCTGGCA	GAGGATGGCCTGGCTGAT
Plin2	GTGGAAAGGACCAAGTCTGTG	GACTCCAGCCGTTCATAGTTG
Plin3	TGTCCAGTGCTTACAACTCGG	CAGGGCACAGGTAGTCACAC
Plin4	TCCTGCTCTGAGGGACCCTT	TCTTGCCTTTGGATTTGGGG
Plin5	TGTCCAGTGCTTACAACTCGG	CAGGGCACAGGTAGTCACAC
Pnpla3	CTCCCTCTCGGCCGTATAAT	AGTCGTGGATGCCCTGGTGT
Pparα	CCTTCTACGCTCCCGACCCA	CCATGTCCATAAATCGGCACCA
Puma	TACGAGCGGCGGAGACAAG	GTGTAGGCACCTAGTTGGGC
Rnf44	AGAGCACAGCTGCGTCCTG	GGGCTCACAACCCGGCA
Scd1	GCCGAGCCTTGTAAGTTCTG	CCTCCTGCAAGCTCTACACC
Sqle	GATGGGCATTGAGACCTTCT	TTTAAAAGAGCCCGACAGGA
Srebp-1c	AACGTCACTTCCAGCTAGAC	AACGTCACTTCCAGCTAGAC
Srebp2	CCCTATTCCATTGACTCTGAGC	GAGTCCGGTTCATCCTTGAC
Syvn1	GCGTCTAGGACCTTGTCCTTT	CCAGAGACCTGTGAACGCTAGG
Tgfβ	GCTGAACCAAGGAGACGG	ATGTCATGGATGGTGCCC
Timp1	AGGTGGTCTCGTTGATTCGT	GTAAGGCCTGTAGCTGTGCC
Tnfα	ACTTCGGGGTGATCGGTCCCC	TGGTTTGCTACGACGTGGGCTAC
Ube2o	GCTCTATGGCCAAGAAGGTGA	CCCTATTTCACTCCCGGCTC
Ulk1	ACCATTGTCTACCAGTGT	AGTGTCTTGTTCTTCTCTAA
Usp10	CCAGTGCCTCCCAAACCCCG	GTCCTCCTGCCGGCCCTTTTC
Wipi1	CACAGGATGGAGGAGAAT	GATGGAGGTAAGGAAGGT

DMSO or tunicamycin (5 μg/mL). Twenty-four hrs later, the cells were stained with BODIPY (*Invitrogen*, D3922, 493/503, 2 μM) using a protocol described by Bo Qiu.[33]

2.8 | Plasma and liver metabolite measurements

Serum cholesterol, triglycerides (TG), and alanine transaminase (ALT) were measured using the commercial kits (*Pointe Scientific* A7510, A7525, and T7532) according to the manufacturer's instructions. For liver TG content, liver tissues were homogenized for total lipid extraction according to Bligh and Dyer (Bligh and Dyer 1959). Total lipids were re-suspended in 400 μL of 100% ethanol. A total of 3 μL were used for the measurement of TG with the TG kit (*Pointe Scientific* T7532).

2.9 | Statistical analysis

All data are reported as Mean ± SD Differences between two groups were assessed by the two-tailed Student's *t* test. Difference between more than two groups was analyzed by ANOVA followed by the Tukey's post hoc testing. *P* value $< .05$ was deemed statistically different.

3 | RESULTS

3.1 | E4BP4 is a novel target induced by ER stress in hepatocytes

We previously reported that insulin induces the *E4bp4* expression via an SREBP-1c-dependent manner and such an induction is critical for de novo lipogenesis in hepatocytes.[27] Whether the *E4bp4* expression is also sensitive to other lipogenic stimuli remains unknown. A recent study reported that the ER stress inducer tunicamycin increases the *E4bp4* expression in pancreatic β-cells and impairs β-cell metabolism and insulin secretion.[29] Given the sensitivity of hepatocytes to ER stress stimuli, we tested whether tunicamycin could induce *E4bp4* by treating both PMHs for indicated time points. Induction of the *Chop* mRNA confirmed the positive response to tunicamycin stimulation in hepatocytes. Meanwhile, the *E4bp4* mRNA was induced as early

as 2 hours following tunicamycin treatment and peaked at 4 hours with an increase by threefold (Figure 1A). Since the saturated fatty acid palmitate was shown to trigger ER stress in hepatocytes,[30] we treated hepatocytes with palmitate and observed a similar elevation of *E4bp4* expression in PMHs (Figure 1B). In contrast, oleic acid (OA), an unsaturated fatty acid, showed no effect on the expression of either *Chop* or *E4bp4* mRNA in mouse hepatocytes (Figure 1C). Lastly, we observed a time-dependent induction of E4BP4 protein in PMHs (Figure 1D). Of note, similar results were observed in the mouse hepatoma Hepa1c1c7 cell line (Figure S1A-D).

To test whether this response is also conserved in the mouse liver, we challenged WT mice with tunicamycin through intraperitoneal injection. Eight and 24 hours post injection, we examined both the protein and mRNA expression of *E4bp4* in the liver. Indeed, liver *E4bp4* mRNA and protein were potently induced by tunicamycin injection along with the induction of GRP78 (Figure 1E,F), a well-known target of acute ER stress response. Collectively, these data support that E4BP4 is a novel target of the ER stress responses in both hepatocytes and the liver.

Interestingly, E4BP4 induction as a novel target of the ER stress response is not limited to only hepatocytes. We also detected the elevated levels of both *E4bp4* mRNA and protein after tunicamycin treatment in the mouse macrophage RAW264.7 cells (Figure S2A,B).

3.2 | HFLMCD diet feeding increases liver E4BP4 expression in WT mice

A variety of diets enriched in saturated fat and cholesterol with certain nutrient depletion have been used to induce hepatic ER stress.[6,11] In particular, high-fat, low methionine, and choline-deficient (HFLMCD) diet has been shown to stimulate hepatocyte ER stress, liver steatosis, and liver inflammation in a relatively short duration.[31] In our hands, feeding with HFLMCD diet for 10 weeks led to elevated levels of serum ALT, liver TG, and liver cholesterol (Figure 2A-C), while liver histology demonstrated clear signs of lipid accumulation by H&E staining and fibrosis by Sirius Red staining (Figure 2D), mimicking human NASH. We also detected a significant induction of hepatic *E4bp4* mRNA of female mice and a remarkable increase of hepatic E4BP4 protein in male mice along with the elevated ER stress marker *Chop* mRNA after 10-week HFLMCD diet (Figure 2E,F and Figure S2C,D). Taken altogether, our data for the first time demonstrate that hepatic E4BP4 is elevated in the liver of mice with HFLMCD diet-induced steatosis and fibrosis.

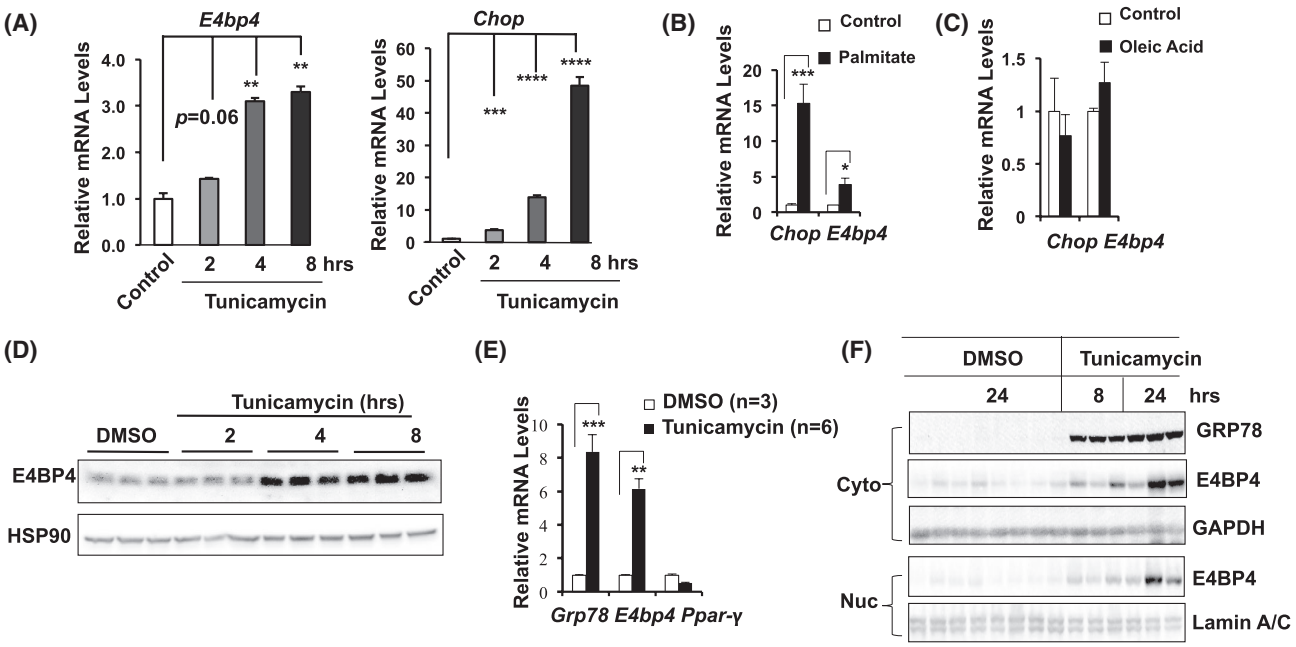

FIGURE 1 ER stress induces *E4bp4* mRNA and protein in primary hepatocytes and mouse liver. A-C, Primary mouse hepatocytes were isolated from WT mice and then, treated with tunicamycin at 5 μg/mL for 2, 4, and 8 hours (A), or palmitate (400 μM) (B) or oleic acid (300 μM) (C) for 24 hours before harvest for mRNA analysis. The mRNA levels of *Chop* and *E4bp4* were examined by RT-qPCR. D, The protein levels of E4BP4 in primary hepatocytes were determined by immunblotting during a time-course study of tunicamycin treatment (E-F) WT male mice were acutely injected with either DMSO (n = 3-7) or tunicamycin (n = 3-6) and liver tissues were harvested at either 8 or 24 hours for both mRNA and protein analysis. The mRNA abundance of *Grp78*, *E4bp4,* and *Ppar-γ* was analyzed by RT-qPCR, whereas the protein levels of E4BP4 and GRP78 were examined by immunoblotting. Data were presented as Mean ± SD At least three independent experiments were performed with similar results

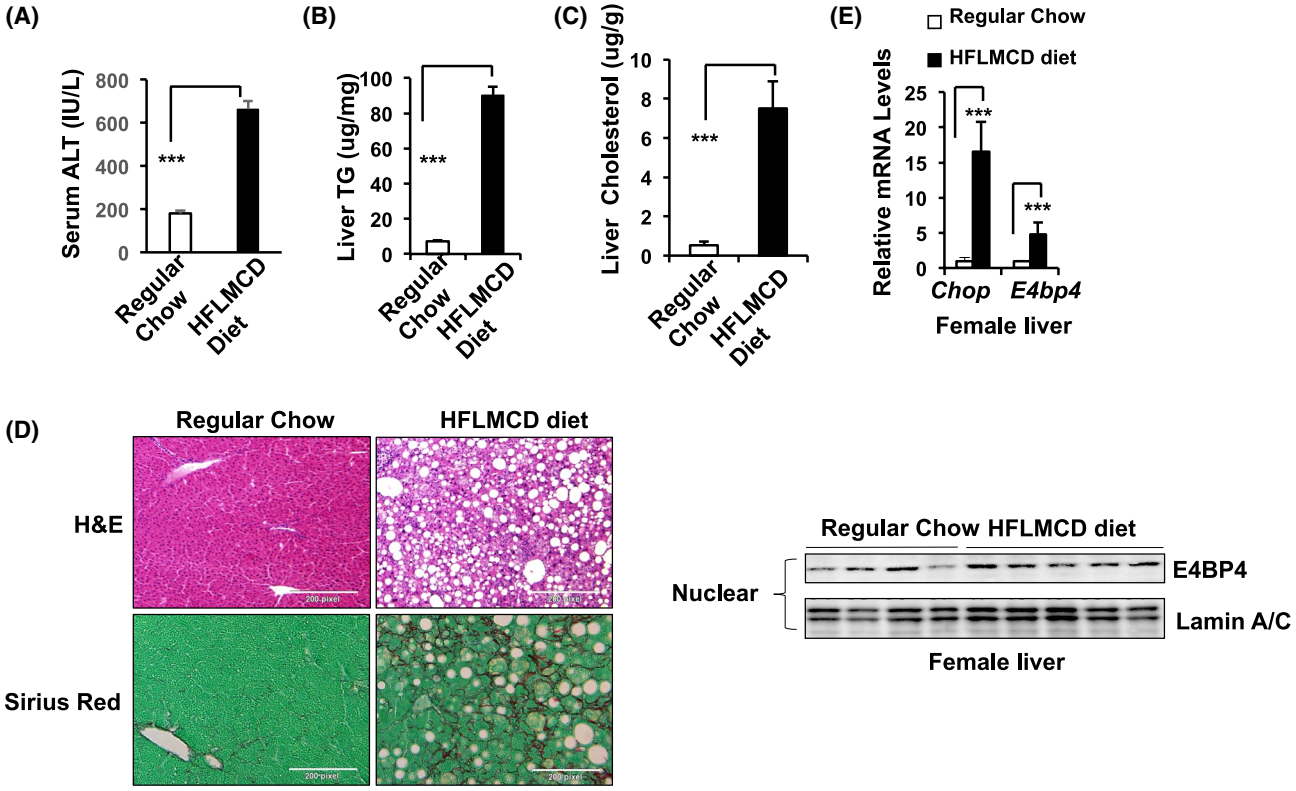

FIGURE 2 HFLMCD diet induces E4BP4 expression in the mouse liver. *C57BL6* female mice were fed either regular chow (n = 4) or HFLMCD diet (n = 8) for 10 wks to induce NASH prior to the following assays: A, serum ALT; B-C, total liver TG & cholesterol; D, liver histology by H&E staining and liver fibrosis by Sirius Red staining; E and F, Liver *E4bp4* mRNA and protein expression in regular chow *vs* HFLMCD diet-fed females. Data were presented as Mean ± SD $^{*}P < .05$, $^{***}P < .001$ by unpaired Student *t* test

3.3 | CHOP is partially required for tunicamycin-induced E4bp4 expression

The transcriptional program upon ER stress has been well characterized in mammalian cells.[1] CHOP acts downstream of the PERK-ATF4 pathway to promote cell death during prolonged ER stress.[32] Hepatic induction of CHOP following tunicamycin injection is necessary for the suppression of genes critical for lipid metabolism.[33] Interestingly, the global *Chop* KO mice fed a diet high in fat, fructose, and cholesterol for 16 wks developed more severe histological features of NASH compared with WT controls.[19] We, therefore, examined whether *Chop* knockdown affects the tunicamycin-induced *E4bp4* in both cultured hepatocytes and the mouse liver. Acute depletion of *Chop* in hepatocytes was achieved by transduction with Ad-shChop and confirmed by RT-qPCR (Figure 3A). In PMHs, tunicamycin induced a robust increase of *E4bp4, Chop, and Grop78* in Ad-shLacZ-transduced controls, whereas *Chop* depletion largely abrogated the *E4bp4* induction without affecting *Grp78* (Figure 3A). A similar loss of E4bp4 induction was observed in the Ad-shChop-injected mouse liver after tunicamycin injection (Figure 3B), suggesting that CHOP is required for the maximal *E4bp4* induction by tunicamycin in hepatocytes and the liver.

Given the role of CHOP as a transcription factor, we hypothesized that CHOP could directly activate the promoter of *E4bp4* in response to tunicamycin. We did find that the ectopic expression of HA-*Chop* induced the luciferase activity driven by the mouse *E4bp4* promoter in a dose-dependent manner in 293A cells (Figure 3C). We previously reported that the E-box element within the proximal region of the *E4bp4* promoter is important for its activation by SREBP-1c.[27] Thus, we tested whether this response element is also required for the CHOP-mediated induction by generating a mutant reporter construct with the deletion of the E-box element. As shown in Figure 3D, Chop overexpression was only able to activate the E4bp4- WT-luc but not the E4pb4-ΔE-box-luc mutant, suggesting that this E-box element is indeed critical for the transcriptional activation of *E4bp4* by CHOP (Figure 3D). However, we were unable to detect direct binding of CHOP to the *E4bp4* promoter by ChIP assay (data not shown), indicating that CHOP might stimulate other transcription factors to activate the *E4bp4* promoter.

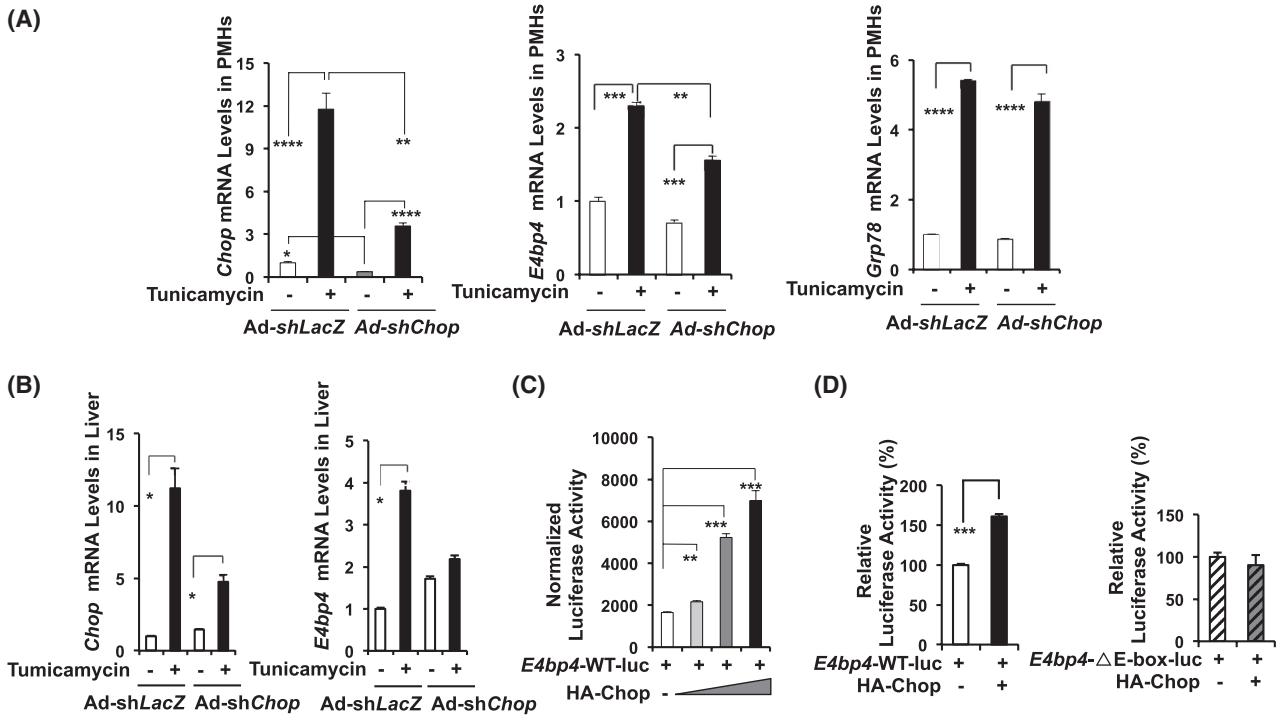

FIGURE 3 *Chop* is critical for the induction of *E4bp4* by tunicamycin in PMHs and the mouse liver. A, PMHs were transduced with AdshlacZ or Ad-shChop before treatment of tunicamycin for 24 hours. The cells were then harvested for mRNA analysis of *E4bp4*, *Chop,* and *Grp78*. B-C, WT mice were tail-vein injected with either AdshLacZ or AdshChop for 10 days before intra-peritoneal injection of DMSO or tunicamycin for 24 hours. The mRNA and protein levels of E4BP4 were analyzed by TR-qPCR and immunoblotting, respectively. C, Activation of the *E4bp4* promoter activity by ectopic expression of *Chop*. 293AD cells were transfected with a luciferase reporter construct driven by the E4bp4-WT promoter plus an increasing amount of the HA-Chop expression construct. The luciferase activity was normalized by the β-gal activity. D, The E-box element in the promoter is required for its induction by CHOP. A total of 293AD cells were transfected with the E4bp4-WT-luc or the E4bp4-ΔE-box-luc mutant plus the HA-Chop expression construct. The luciferase activity was normalized by the β-gal activity. Data were presented as Mean ± SD. $*P < .05$, $***P < .001$ by unpaired Student *t* test. The in vitro experiments were repeated at least twice with similar results

3.4 | E4BP4 is required for tunicamycin-induced lipid droplet accumulation in cultured hepatocytes

Both acute and chronic ER stress potently impact hepatic lipid homeostasis.[10] For example, administration of tunicamycin into WT mice was reported to influence lipid synthesis and breakdown, leading to massive liver steatosis.[5] We previously reported that E4BP4 mediates de novo lipogenesis downstream of the insulin signaling in hepatocytes.[27] To test whether E4BP4 is involved in the tunicamycin-induced lipid accumulation in hepatocytes and the liver, we generated *E4bp4* liver-specific knockout (*E4bp4-LKO*) mice by crossing *E4bp4*$^{flox/flox}$ mice with *Alb-Cre* transgenic mice and validated the liver-specific deletion of E4BP4 (Figure 4A). Next, we challenged both *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* PMHs with tunicamycin, and analyzed the impact of *E4bp4* deficiency on lipid droplet formation and TG content in hepatocytes. As shown by BODIPY staining,[28] overnight incubation of tunicamycin markedly increased both the number and size of lipid droplets in *E4bp4*$^{flox/flox}$ hepatocytes. However, tunicamycin-induced lipid droplets were nearly abolished in the *E4bp4-LKO* PMHs (Figure 4B), consistent with the reduced levels of TG content in tunicamycin-treated *E4bp4-LKO vs E4bp4*$^{flox/flox}$ PMHs (Figure 4C). These observations support that E4BP4 is indeed required for lipid droplet accumulation in response to tunicamycin-induced ER stress in hepatocytes.

Lipid homeostasis in hepatocytes is a dynamic process coordinated via lipid uptake, lipid synthesis, fatty acid oxidation, and lipid export.[34] To explore the downstream targets of E4BP4 during lipid accumulation in hepatocytes, we measured the mRNA expression of common lipid metabolic pathways in PMHs. On the one hand, tunicamycin elevated the expression of *Fasn* while repressing *Cpt1a* in *E4bp4*$^{flox/flox}$ but not *E4bp4-LKO* PMHs (Figure 4D). On the other hand, there were no differences in the expression of genes involved in autophagy, lipolysis, and lipid export between those two groups (Figure S3). Chronic ER stress also has been reported to induce hepatocyte apoptosis.[35,36] We, therefore, examined whether E4BP4 is required for this process. As shown in Figure S5, tumicamycin treatment

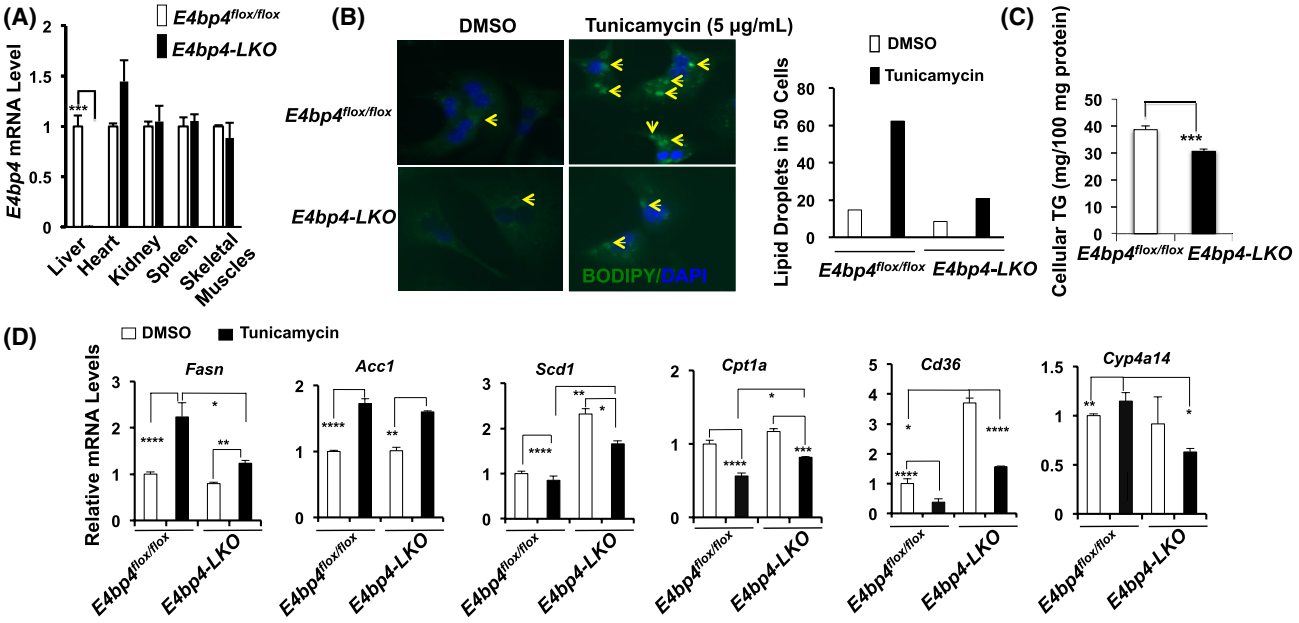

FIGURE 4 Hepatic *E4bp4* is required for tunicamycin-induced accumulation of lipid droplets in PMHs. A, Validation of liver-specific *E4bp4* deficiency in various tissues isolated from both *E4bp4*$^{flox/flox}$ (n = 3) and *E4bp4-LKO* (n = 3) mice by RT-qPCR. PMHs were isolated from *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* mice and treated with tunicamycin overnight prior to BODIPY staining for lipid droplets and RNA extraction for RT-qPCR. B, Image of lipid droplet formation after tunicamycin treatment; lipid droplets in 50 cells were countered per condition and presented in the bar graph. C, Cellular triglycerides (TG) content in tunicamycin-treated *E4bp4*$^{flox/flox}$ or *E4bp4-LKO* PMHs. D, Lipid metabolic genes assessed in PMHs of *E4bp4-LKO* mice following tunicamycin treatment

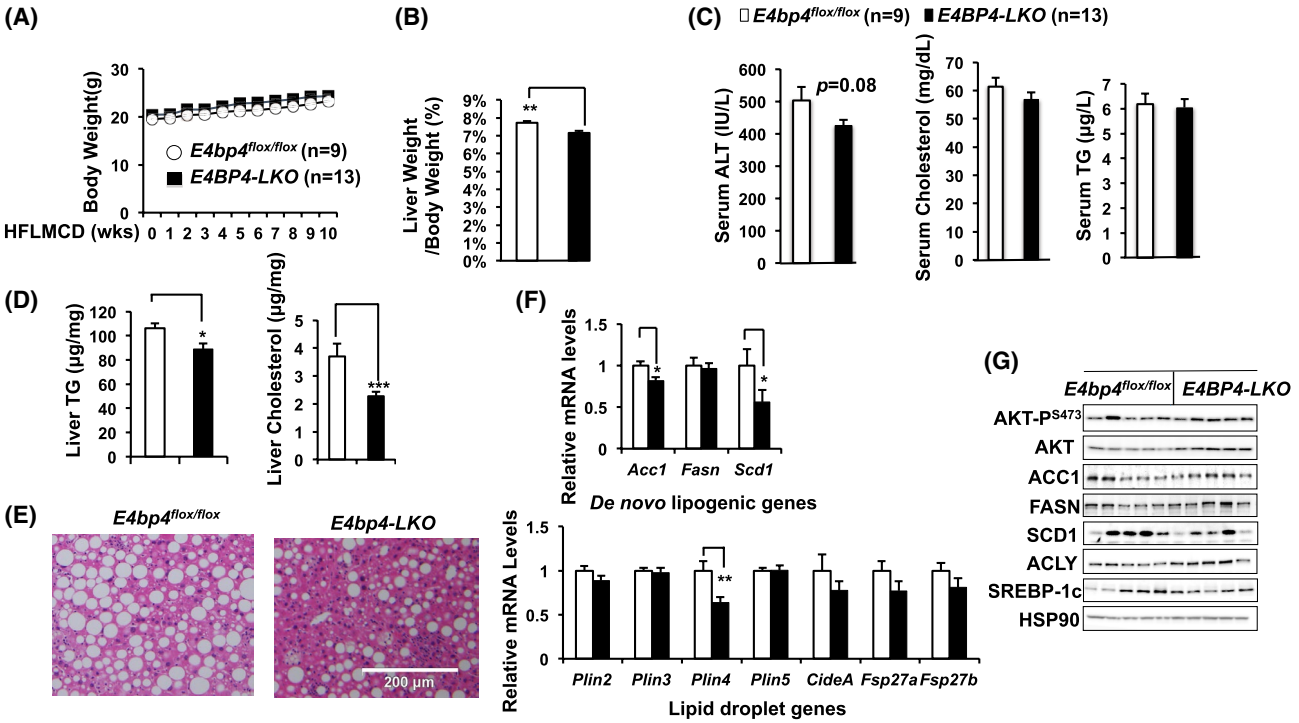

FIGURE 5 Loss of hepatic *E4bp4* protects mice from HFLMCD diet-induced lipid accumulation in the liver. Both *E4bp4* $^{flox/flox}$ and *E4bp4-LKO* were fed HFLMCD diet for 10 weeks before sacrifice for the following assays: A and B, body weight and liver weight/body weight ratio; C, serum ALT, TG, and cholesterol; D, liver TG and cholesterol; E, Liver histology by H&E staining; F, Quantification of lipid metabolic gene expression in the liver. G, Immuoblotting of AKT-P^{S473}, ATK, lipogenic enzymes, and SREBP-1c in the liver. *$P < .05$; **$P < .01$, compared with *E4bp4*$^{flox/flox}$ group. All data are expressed as Mean ± SD

significantly induced pro-apoptotic genes including *Noxa* and *Puma* in *E4bp4*$^{flox/flox}$ but not *E4bp4-LKO* PMHs, indicating that E4BP4 induction may contribute to ER stress-induced apoptosis. Together, these findings reveal a broad role of E4BP4 in lipid metabolism and hepatocyte apoptosis following ER stress.

3.5 | Loss of hepatic *E4bp4* protects against HFLMCD diet-induced lipid accumulation

Given the important role of ER stress in hepatocytes during NAFLD[2] and the elevated E4BP4 expression in the liver of the HFLMCD diet-induced ER stress mouse model (Figure 2), it was logical to examine whether manipulation of hepatic E4BP4 impacts lipid metabolism and NAFLD in vivo. We first compared *E4bp4*$^{flox/flox}$ *vs E4bp4-LKO* mice on regular chow following fasting overnight and refeeding for 12 hours. The reason we chose to refeed mice is because the E4BP4 mRNA and protein tend to be potently induced by refeeding.[37] Body weight, TG, and cholesterol in serum/liver, as well as serum ALT all turned out to be comparable between the two groups of mice (Figure S6), supporting that hepatic E4BP4 is dispensable in liver lipid metabolism under the normal physiological condition.

Next, we examined the impact of hepatic deletion of *E4bp4* on lipid metabolism following HFLMCD diet. Upon 10-wk HFLMCD feeding, both *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* mice showed similar body weight gain (Figure 5A). However, the ratio of liver weight/body weight was significantly reduced in *E4bp4-LKO* mice (Figure 5B). Serum ALT, the classical marker for liver injury, was about 20% lower in *E4bp4-LKO* mice (Figure 5C). Despite comparable levels of serum TG and cholesterol in both groups of mice (Figure 5D), *E4bp4-LKO* mice accumulated significantly less TG, total cholesterol, and lipid droplets in the liver by H&E staining (Figure 5D,E). In summary, all these data suggest that *E4bp4-LKO* mice are protected from HFLMCD-induced liver steatosis and cholesterol accumulation.

To further explore how *E4bp4* deficiency renders mice resistant to liver steatosis, we measured the genes of major lipid metabolic pathways in both groups of mice. In comparison with *E4bp4*$^{flox/flox}$ mice, *E4bp4-LKO* mice showed significantly lower expression of genes involved in de novo lipogenesis (*Acc*1 and *Scd1*) and lipid droplet formation (*Plin4*) in the liver (Figure 5F). At the protein levels, SCD1 protein levels were markedly reduced in the liver of *E4bp4-LKO* mice (Figure 5G). Of note, liver-specific *Scd1* knockout mice showed less lipid accumulation in the liver in response to high-carbohydrate diet.[38,39] In contrast, no significant differences were detected in the genes involved in lipolysis, cholesterol biosynthesis, fatty acid oxidation, and fatty acid uptake in the liver between those two groups of HFLMCD diet-fed mice (Figure S7).

Since NASH is associated with increased liver inflammation and fibrosis, we then compared the expression of inflammatory and fibrotic markers in the liver between *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* mice following HFLMCD. The mRNA levels of inflammatory cytokines (*Tnfα*, *Il-1β*, *Mcp1,* and *F4-80*) as well as fibrotic genes (*Cola1*, *αSma, and Tgfβ*) in the liver were comparable between the two groups (Figure S8A,B), in agreement with the similar staining pattern of the liver tissues by Masson's Trichrome staining (Figure S8C). These data suggest that under the diet-induced ER stress condition, hepatic *E4bp4* deficiency protects mice from lipid accumulation without impacting inflammation and fibrosis in the liver.

3.6 | The role of AMPK suppression in E4BP4-driven lipid accumulation in hepatocytes

To further uncover the molecular mechanisms underlying the reduced expression of de novo genes and lipid accumulation in the liver of HFLMCD diet-fed *E4bp4-LKO* mice, we focused on the energy-sensing kinase AMPK, a critical regulator of lipid homeostasis in the liver.[40] It has been well recognized that the AMPK-dependent phosphorylation of ACC potently inhibits de novo lipogenesis in the liver,[41] whereas the AMPK-dependent phosphorylation of ATGL promotes lipolysis.[42] Several in vivo studies revealed the beneficial effects of chronic AMPK activation on diet-induced liver steatosis.[43,44] Whether hepatic *E4bp4* deficiency could affect the AMPK pathway in hepatocytes and subsequently diet-induced lipid accumulation in the liver is currently unknown.

AMPK activation is marked by AMPK phosphorylation at T172.[45] We first examined the effect of *E4bp4* deficiency on AMPK-P^{T172} in the liver of HFLMCD diet-fed mice. Indeed, the AMPK-P^{T172} levels were elevated in the liver of *E4bp4-LKO* mice following HFLMCD diet feeding (Figure 6A). Similarly, we observed increased levels of AMPK-P^{T172} in *E4bp4-LKO* PMHs (Figure 6B), supporting that E4BP4 regulates AMPK-P^{T172} in a cell-autonomous manner in hepatocytes. Conversely, adenoviral over-expression of E4BP4 substantially reduced not only the basal but also the 2-Deoxy-D-glucose-induced AMPK-P^{T172} in WT mouse hepatocytes (Figure 6C). These findings collectively support that E4BP4 acts as a negative regulator of the AMPK pathway in hepatocytes.

To test how hepatic E4BP4 regulates the AMPK pathway, we examined whether E4BP4, as a transcription factor, affects the expression of hepatic *Ampk* subunits. The mRNA levels of the major AMPK subunits were comparable in the liver of *E4bp4*$^{flox/flox}$ *vs E4bp4-LKO* mice (Figure S9A). However, at the protein level, AMPKβ1 was

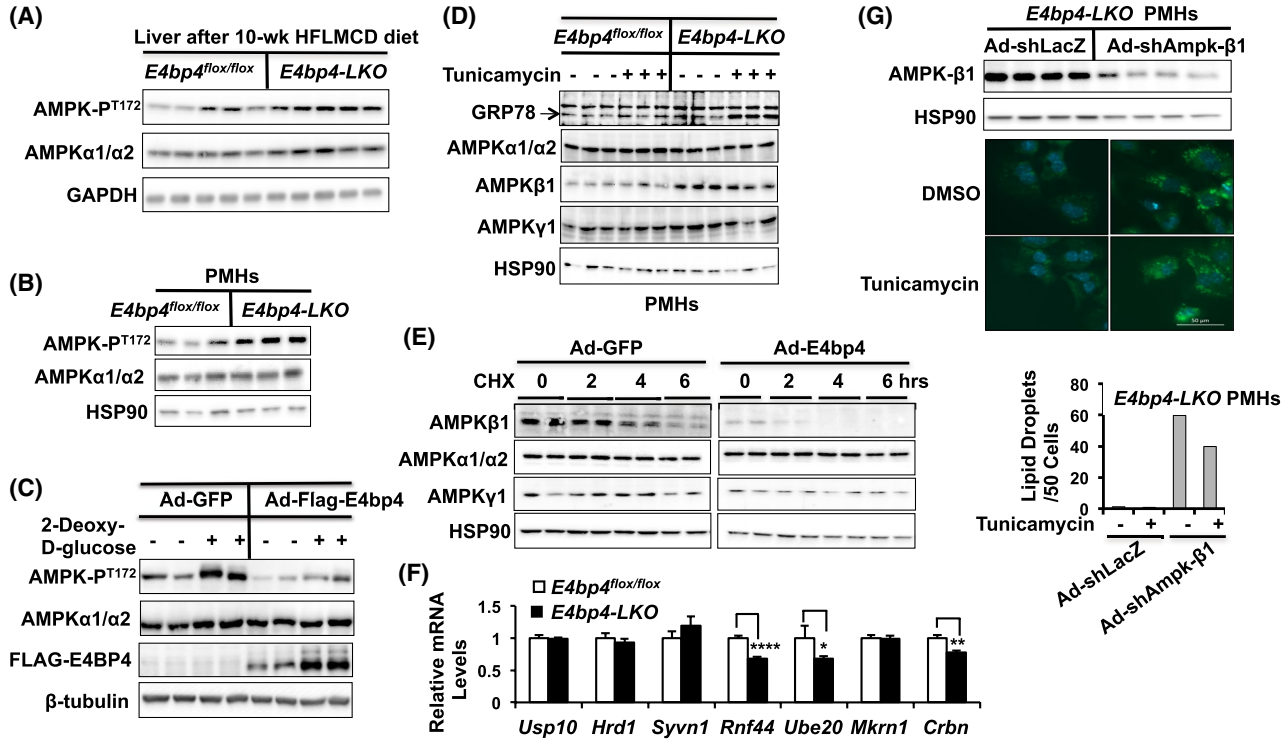

FIGURE 6 E4BP4 targets the AMPK-β1 subunit to mediate the tunicamycin-induced lipid accumulation in hepatocytes. A, Comparison of AMPK activation by anti AMPK-phosphorylation at T172 in the liver *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* mice following HFLMCD feeding. B, The levels of AMPK-P^{T172} in PMHs from *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* mice. C, Adenoviral over-expression of E4BP4 in WT PMHs reduces the basal and 2-Deoxy-D-glucose-induced AMPK-P^{T172} level. D, The protein abundance of AMPK subunits in *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* PMHs by immunoblotting. D, Effect of E4BP4 overexpression on the stability of AMPK-β1. E, Effect of acute depletion of *Ampk-β1* by adenoviral shAmpk-β1 on lipid droplets in *E4bp4-LKO* PMHs following tunicamycin treatment. The knockdown efficiency of Ad-shAmpk-1 was confirmed by immunblotting with anti-AMPK-β1. F, The mRNA levels of candidate ubiquitin E3 ligases in the liver of *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* mice following HFLMCD feeding. *$P < .05$; **$P < .01$, compared with *E4bp4*$^{flox/flox}$ group. All data are expressed as Mean ± SD

markedly elevated in both *E4bp4-LKO* PMHs (Figure 6D) and the liver of *E4bp4-LKO* mice (Figure S9B). These data suggest that E4BP4 might control the proteolysis of AMPKβ1 in hepatocytes.

AMPKβ1 is the scaffolding subunit in the intact AMPK kinase complex in mouse hepatocytes.[46] Interestingly, we found that, under the tunicamycin-induced ER stress, only the AMPKβ1 protein abundance was reduced (Figure S9D). To directly test the impact of E4BP4 on the AMPK-β1 degradation, we measured the protein half-life of AMPKβ1 in Ad-E4bp4-transduced Hepa1 cells in cycloheximide chase analysis. In cells transduced with Ad-GFP, AMPKβ1 had a half-life of about 3 hours. However, its half-life was shortened to less than 2 hours in cells transduced with Ad-E4bp4 (Figure 6E). This observation was consistent with the formation of AMPKβ1-polyubiquitin conjugates in Ad-E4bp4-transduced PMHs (Figure S10A). We previously reported that E4BP4 forms a complex with the nuclear SREBP-1c and promotes lipogenic gene expression in response to insulin signaling.[27] We, therefore, checked whether SREBP-1c over-expression affects the AMPKβ1 protein abundance just like E4BP4. As shown in Figure S10B, adenoviral SREBP-1c overexpression showed no effect on the AMPKβ1 abundance in cultured hepatocytes, suggesting that the E4BP4 action on the AMPKβ1 stability is likely to be independent of SREBP-1c in hepatocytes.

How does E4BP4 controls the AMPKβ1 proteolysis? We speculate that E4BP4 may control the expression of critical regulators that either promote or suppress the stability of AMPKβ1 in hepatocytes. It has been reported that the ubiquitin E3 ligase MKRN targets the AMPKα subunits for degradation, whereas the ubiquitin-specific protease USP10 protects the AMPK complex from proteolysis.[47-49] We screened the expression of the reported regulators of the AMPK complex by RT-qPCR and found that several AMPK-specific E3 ligases including *Rnf44*, *Ube2e*, and, *Celebron*[49-51] were significantly downregulated in the liver of *E4bp4-LKO* mice following HFLMCD diet feeding (Figure 6F). Thus, it is likely that E4BP4 could suppress the AMPKβ1 protein stability via the upregulation of these E3 ligases.

Since AMPKβ1 is essential for the integrity of the AMPK protein complex,[46] we reasoned that the depletion of *Ampk-β1* by shRNA could lead to the disassociation of the AMPK kinase complex and subsequently suppress lipid

droplet formation in *E4bp4-LKO* PMHs. To test this hypothesis, we challenged *E4bp4-LKO* PMHs with tunicamycin after depleting *Ampkβ1* with Ad-sh*Ampk-β1* (validated by immunoblotting in Figure 6G) and then, performed lipid droplet staining. As shown in Figure 6G, no increase of lipid droplets was detected in Ad-shLacZ-transduced *E4bp4-LKO* PMHs after tunicamycin treatment. However, knockdown of *Ampk-β1* led to a drastic induction of lipid droplets in *E4bp4-LKO* PMHs in the presence or absence of tunicamycin treatment. In summary, our data support that E4BP4 is likely to target the AMPKβ1 subunit to promote lipid accumulation in hepatocytes in response to ER stress inducers.

4 | DISCUSSION

Previously, we reported that the b-ZIP transcription factor E4BP4 could be induced in the liver via actions of nutritional and hormonal signaling to regulate hepatic lipid metabolism.[22,27] Here we demonstrate that ER stress potently induces E4BP4 in the liver and hepatocytes to cause lipid accumulation in the liver. Depletion of hepatic *E4bp4* not only protects against tunicamycin-induced lipid droplet accumulation in cultured hepatocytes but also reduces liver steatosis in a diet-induced NAFLD mouse model. At the mechanistic level, we found that CHOP is required for the maximal induction of E4BP4 by ER stress in hepatocytes. We discovered that *E4bp4* deletion impairs hepatic de novo lipogenesis and lipid accumulation likely through promoting the AMPK activity in hepatocytes following ER stress. Our findings point to E4BP4 as a novel target for treating liver diseases associated with ER stress (Figure S10C).

It has been established that ER stress promotes NAFLD by altering hepatic lipid metabolism.[4,10] Specific effectors of ER stress pathway and their downstream signaling molecules have been examined in cell cultures and animal models for their roles in the ER stress-induced lipid accumulation in hepatocytes. A PERK inhibitor was found to improve liver steatosis and insulin sensitivity in an animal model of NAFLD.[52] In contrast, deletion of *Ire1* leads to spontaneous liver steatosis,[53] indicative of the unique functions of specific ER stress mediators in regulating lipid metabolism. In the current study, we obtained direct evidence from *E4bp4-LKO* PMHs to support the essential role of E4BP4 in tunicamycin-induced lipid accumulation in hepatocytes. A similar observation was also made in the liver of HFLMCD challenged *E4bp4-LKO* mice. To investigate the impact of hepatic *E4bp4* deficiency with a stronger relevance to advanced human NAFLD in the future, we intend to feed *E4bp4-LKO* mice a diet rich in saturated fat, cholesterol, and sugars.[54] Recently, the Koo group observed reduced E4BP4 in the liver of either 27-week high-fat (60% calories from fat) diet-fed WT or *ob/ob* mice and provided evidence in support of E4BP4 as a negative regulator of hepatic gluconeogenic genes.[25] In our study, both tunicamycin injection and 10-week HFLMCD (45% calories from fat) diet feeding potently induce the mRNA and protein levels of E4BP4 in the liver of *E4bp4*$^{flox/flox}$ mice. We speculate that genetic backgrounds, the type of nutritional stress, and feeding regimen may all contribute to the differences between the studies, highlighting the complexities in the E4BP4-regulated metabolic responses in the liver.

In our study, we examined lipid metabolism genes to assess how E4BP4 impacts lipid metabolism following ER stress. Consistent with our previous studies,[27] several de novo lipogenic genes including *Fasn* and *Acc1* were reduced in the absence of *E4bp4* in hepatocytes. However, *E4bp4* deficiency did not seem to impact fatty acid oxidation, lipid transport, and lipolysis gene expression. We speculated that hepatic *E4bp4* deficiency might repress the activity of lipid metabolic enzymes via the AMPK pathway without significantly affecting the mRNA levels of these lipid metabolic enzymes. Our data indeed show that inhibition of the AMPK pathway by acute knockdown of *Ampk-β1* restores lipid droplet formation in *E4bp4-LKO* PMHs. AMPK is a master regulator of multiple metabolic pathways and have been targeted in treating obesity, insulin resistance, type 2 diabetes, and NAFLD.[40,55,56] One of our novel findings is that E4BP4 is both necessary and sufficient to inhibit the cellular AMPK activity in hepatocytes. *E4bp4*-deficiency in hepatocytes enhances the levels of AMPK-P^{T172}, the hallmark of AMPK activation, whereas *E4bp4* overexpression inhibits AMPK-P^{T172}. The Sternberg's group proposed three potential mechanisms by which AMPK activation may improve NAFLD: (1) the suppression of hepatic de novo lipogenesis; (2) the enhancement of fatty acid oxidation; and (3) the improvement of the mitochondrial function/integrity in adipose tissue.[40,56,57] Surprisingly, despite the reduced ATP content in the livers of patients with NAFLD, hepatic AMPK activity was actually reduced, suggesting that AMPK activation could be regulated independently of ATP levels in the liver during NAFLD.[56,58] Our data point out that E4BP4 could be a critical factor that links nutritional stress and AMPK suppression in hepatocytes during NAFLD. Therefore, the suppression of E4BP4 may be a valid avenue to activate hepatic AMPK activity for the treatment of NAFLD.

So, how does E4BP4 suppress the AMPK signaling in the liver? There has been extensive research on how the intracellular ADP:ATP ratio activates AMPK.[57] However, very little is known about how the changes of the major components of the AMPK complex affect its activity. We found that the mRNA abundance of *Ampk* subunits was similar in the liver between *E4bp4*$^{flox/flox}$ and *E4bp4-LKO* mice (Figure S9A). In contrast, the protein level of the AMPKβ1 subunit was elevated in both *E4bp4-LKO* PMHs and liver tissues. Moreover, E4BP4 overexpression enhances the ubiquitination of AMPKβ1 and accelerates the degradation

of the AMPKβ1 subunit in hepatocytes. Therefore, E4BP4 is likely to inhibit hepatic AMPK pathway by enhancing the AMPKβ1 ubiquitination and degradation. So far, the AMPK subunits were shown to undergo the E3 ligase-mediated ubiquitination and degradation. A case in point, the lipid droplet-binding protein CIDEC was reported to interact with the AMPKα subunits and promote its ubiquitination-dependent degradation during adipogenesis.[59] Recently, several E3 ligases were reported to modulate the protein stability of the AMPK complex, including MKRN, CRBN, RNF44, and UBE2O.[47,49-51] MKRN1 was found to directly target the AMPK-α subunits for ubiquitination and degradation.[47] MKRN-null mice showed chronic AMPK activation in both liver and adipose tissues and were protected against diet-induced metabolic dysfunction. RNF44 promotes the degradation of AMPKα1 in melanoma cells in response to arginine deprivation.[50] The E3 ligase CRBN binds to the AMPK complex and degrades the AMPKα subunits in multiple cell types.[49] The Song group reported that UBE2O acts as a ubiquitin E3 ligase for the AMPK-α2 subunit in skeletal muscles.[49] Intriguingly, we found that the mRNA levels of *RNF44, CRBN,* and *Ube2O* but not MKRN were significantly reduced in the liver of *E4bp4-LKO* mice. Our future study will determine whether these E3 ligases are involved in the regulation of the AMPKβ1 stability downstream of E4BP4 induction in hepatocytes in response to chronic ER stress.

ER stress in macrophages is important for inflammation and the inflammasome activation. Interestingly, we observed that, in the RAW264.1 macrophage cell line, tunicamycin also potently induces *E4bp4* mRNA and protein expression (Figure S2), suggesting that the ER stress-induced E4BP4 pathway could be conserved in multiple cell types. It has been reported that the ER stress pathway is activated in Kupffer cells during NAFLD, resulting in the M1-type polarization of macrophages and subsequently increased liver injury.[60] Even though the essential role of E4BP4 in NK cell development was widely reported,[23] so far, the contribution of E4BP4 in ER-stressed macrophages to NAFLD remains unknown. We will explore the role of E4BP4 in Kupffer cells during the pathogenesis of NAFLD by generating a mouse model with the macrophage-specific deletion of *E4bp4* for future study.

One important finding in our study is the gender-specific protection against HFLMCD-induced NASH. Only female *E4bp4-LKO* mice were protected from TG and cholesterol accumulation in the liver even although hepatic E4BP4 was elevated in both female and male mice following 10-week HFLMCD diet. It has been previously recognized that NAFLD is a sexually dimorphic disease and the metabolic impact of sex hormones is likely to account for the differences between genders.[61] In a human study, over 1000 genes display a sex bias in their expression in the liver with the top biological pathways related to lipid metabolism.[62] Currently, there are no data suggesting a crosstalk between E4BP4 and the estrogen signaling in the liver. For future study, we will identify the gender-specific downstream targets of E4BP4 that might regulate lipid metabolism and the AMPK signaling in a sexually dimorphic manner.

In summary, we have shown that both the *E4bp4* mRNA and protein are highly inducible by ER stress signals in cultured hepatocytes and the mouse liver. We also showed that hepatic E4BP4 is required for lipid accumulation in response to ER stress signals. *E4bp4* liver-specific knockout mice are partially protected from hepatic lipid accumulation and injury induced by HFLMCD diet, likely due to the enhanced AMPK pathway. Furthermore, E4BP4 might reduce the AMPK-β1 stability in the liver to inhibit the AMPK pathway in response to chemical or diet-induced ER stress possibly by upregulating AMPK-specific E3 ligases. Taken together, our data reveal a novel molecular link between E4BP4 and ER stress-induced hepatic lipid accumulation during the pathogenesis of NAFLD and indicate that the suppression of hepatic E4BP4 activation might be a potential therapeutic avenue for treating NAFLD.

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CONFLICT OF INTEREST

The authors claim no conflict of interest.

AUTHOR CONTRIBUTIONS

M. Yang carried out all the treatment, tissue harvesting, immunoblotting, RT-qPCR, and metabolic assays of *E4bp4-LKO* mice with the assistance of D. Zhang, Z. Zhao, O. Shabandri, and M. Yang isolated PMHs and performed BODIPY staining and quantification; X. Tong, M. Yang, and Z. Zhao generated all the expression vectors and recombinant adenoviruses for in vitro and in vivo experiments; M. Yang and M. Saint-Sume did the luciferase assays and proofread the manuscript; X. Tong performed the tail vein injection of mice; K. Zhang provided mouse liver samples for Figure 1F; L. Yin did the cellular and biochemical experiments for AMPK analysis; L. Yin and X. Tong supervised the project and wrote the manuscript with inputs from all the other authors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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